Fate of zearalenone, deoxynivalenol and deoxynivalenol-3-glucoside during malting process

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Abstract

Malting was simulated using two different batches of barley as raw material: a naturally contaminated batch and laboratory inoculated (with a deoxynivalenol (DON) and zearalenone (ZEA) producing *Fusarium graminearum* strain) one. Up to three contamination levels were prepared, every process being carried out in triplicate. A significant washout effect on DON was observed by the end of the first water phase (between 22.4 and 34 % reduction) with an even more pronounced reduction (up to 75 % decrease) by the end of the steeping process. ZEA content remained almost unchanged (no significant difference between the initial and the final concentration). Germination was characterized by an increase in all the three toxins (ZEA, DON and DON-3-G) concentrations, however showing a decreasing trend in the last 24 h of the stage, compared to the first day of germination. Kilning lead to a significant reduction of DON in the naturally contaminated batch (46.6 and 78.8%), nevertheless an increase in all other toxins and contamination levels was observed.

Keywords: Deoxynivalenol, deoxynivalenol-3-glucoside, zearalenone, HPLC-DAD/FLD, malting.

1 Introduction

Barley was domesticated, for the first time, in the southern part of Fertile Crescent (Israel-Jordan) about 8000 BC (Badr et al., 2018) and in about 5000 BC the first records exist about malting and brewing practices by early societies (Meussdoerffer, 2009). Malting process is a crucial stage in brewing and barley quality plays the most important role.

Fusarium Head Blight disease (FHB), which is highly occurring on barley crops, is an important issue for farmers and brewers, firstly, because the disease drastically affects grains' quality (*e.g.* germination energy, nutrients content) and secondly, because it is usually accompanied with mycotoxins accumulation. The main species responsible for FHB are *Fusarium graminearum, Fusarium avenaceum* and *Fusarium culmorum* (Hietaniemi et al., 2016). The predominant mycotoxins contaminating malting barley are deoxynivalenol (DON), zearalenone (ZEA), nivalenol (NIV), T-2 and HT-2 toxins, which may induce neurotoxic, teratogenic, immunosuppressive, oestrogenic and carcinogenic effects in case of ingestion, inhalation or skin contact (Pestka, 2007; Zinedine, Soriano, Moltó, & Mañes, 2007). A previously published review found DON and ZEA as the most commonly occurring mycotoxins on malting barley crops worldwide (Pascari, Ramos, Marín, & Sanchís, 2018).

Besides mycotoxins co-occurrence, a transformation of the original mycotoxins molecule into a different structure is possible which might result unreported because of its undetectability by traditional analytical methods. Deoxynivalenol-3-glucoside (DON-3-G) is the most common modified form of DON as a result of plant detoxification mechanism catalysed by glucosyltransferase enzyme (Freire & Sant'Ana, 2018). Also, DON-3-G formation during germination process might be the result of the activation of starch hydrolysing enzymes, such as amylases, although in a quite low rate due to a lower incidence of DON in the endosperm where starch is stored (Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012). However, after ingestion, the digestive tract's hydrolases tend to break the glucosyl bound and DON is freed in the body (Berthiller et al., 2013). The abovementioned facts corroborate the need for a deeper study of mycotoxins fate during processing in order to evaluate the possible risk for consumers and to revise the current legal limits for mycotoxins and their derived forms.

There are various researchers dedicated to study malting barley infestation with *Fusarium* spp. and their mycotoxins (Beccari, Caproni, Tini, Uhlig, & Covarelli, 2016; Medina et al., 2006; Piacentini, Savi, Pereira, & Scussel, 2015). After proving fungal activity during germination process, by one hand, and the discovery of modified mycotoxins, by the other,

researchers started to question the fate of mycotoxins during malting (Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Kostelanska et al., 2011; Pietri, Bertuzzi, Agosti, & Donadini, 2010; Vaclavikova et al., 2013).

The aim of the present work is to study the fate of DON, DON-3-G and ZEA, during malting process, focusing on naturally contaminated barley. Moreover, a comparison with mycotoxins' fate during malting process of laboratory infested barley was performed. Also, different concentration levels were taken into consideration for the study of the evolution of mycotoxins level throughout the process. The results will also allow to identify the stages at which significant changes in mycotoxins' concentration take place.

2 Materials and methods

2.1 Chemicals and reagents

Deoxynivalenol, deoxynivalenol-3-glucoside (50.4 µg/mL) and zearalenone standards were purchased from Romer Labs (Tulln, Austria) and stored at -18 °C. Solid standards (deoxynivalenol and zearalenone) were resuspended in methanol before storage and stock solutions of 758 and 844 µg/mL for DON and ZEA, respectively, were obtained.

Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol, acetonitrile (HPLC grade), potassium di-hydrogenophosphate and chloramphenicol were purchased from Scharlab (Sentmenat, Spain). Sodium chloride, disodium hydrogen phosphate, potassium di-hydrogen phosphate, glucose and potassium chloride were purchased from Fischer Scientific (New Jersey, USA). Peptone bacteriological was bought from Biokar Diagnostics (Allonne, France). Magnesium sulfate heptahydrate was purchased from Probus SA (Badalona, Spain), agar powder from VWR Prolabo (Leichestershire, UK), rose Bengal from ICN Biomedicals (Ohio, USA) and dichloran was obtained from Sigma Aldrich (St. Louis, Missouri, USA).

2.2 Barley

Uncontaminated malting barley (*Hordeum vulgare*) from the 2017 harvest was supplied by the malting plant Malteria la Moravia S.L (10 kg). (Bell-lloc d'Urgell, Spain). The absence of DON, DON-3-G and ZEA in the uncontaminated batch was confirmed by HPLC-DAD/FD system. Barley grains were stored in a cool and dry place until malting. Naturally contaminated malting (15 kg), *Hordeum vulgare* of the same harvest year as the uncontaminated batch but a different region in Spain, already containing DON (676 µg/kg), DON-3-G (170µg/kg) and ZEA (55 µg/kg), was taken from a rejected batch in a food company, due to its DON content.

2.3 Barley mycobiota

In order to evaluate the initial mycobiota of barley kernels from the two sources (uncontaminated barley and naturally contaminated with DON), 600 kernels of each were incubated on Dichloran Rose Bengal Chloramphenicol Agar medium (DRBC): 300 kernels directly placed onto Petri dish (5 kernels per dish) and another 300 kernels were previously disinfected by submerging them in a 2g/100 mL sodium hypochlorite solution for 1 min, followed by double immersion in sterile water of 1 min each (also plated 5 kernels per dish) (Andrews, Pardoel, Harun, & Treloar, 1997). The incubation temperature was 25 °C, during 7 d. After incubation they were examined for fungal growth, and the moulds present on the kernel were identified to genus level using the methods of Pitt & Hocking (1997).

2.4 DON and ZEA contamination of the inoculated barley

A strain of *F. graminearum* (F.46) (producer of DON and ZEA) from the Food Technology Department's culture collection was used for fungal contamination of the grain and mycotoxin production. Barley grains were disinfected according to Andrews, Pardoel, Harun, & Treloar (1997). Briefly, 500 g of grains were immerged into 0.4 g/100 mL chlorine solution for 2 min and then abundantly rinsed with sterile distilled water. Then, the grains were placed in a hermetically closed sterile ISO bottle and left overnight with a small amount of water (approximately 340 mL/kg) at 4 °C to achieve a water activity of 0.99 (Aqualab Series 3 TE, Decagon Devices Inc., Washington, USA). Afterwards, the humidified barley was aseptically transferred to Petri dishes and 1 mL of spore suspension of *F. graminearum* (10^6 spores/mL) was transferred on each dish. Petri dishes with barley were then incubated at 25 °C for 30 d. Afterwards, the contaminated grains were dried at 40 °C for 24 h, homogenized and DON, DON-3-G and ZEA levels were determined. The obtained concentrations were: 780.5 µg/kg for DON and 685.7 µg/kg for ZEA. DON-3-G was not detected.

2.5 Experimental design

Experiments were carried out using naturally contaminated barley. Moreover, an additional experiment was performed using barley inoculated in the laboratory in order to assess the impact of two sources of contamination on mycotoxins' concentration change during malting. Before malting, contaminated and uncontaminated barley were mixed to achieve two and three concentration levels for naturally contaminated (62 and 100 % of contaminated kernels added) and laboratory inoculated barley batches (5.3, 11.4 and 22.8 % of contaminated grains added), respectively. All tested contamination levels were prepared per triplicate. Table 1 shows the data concerning the prepared samples.

2.6 Laboratory scale malting

Laboratory scale malting was performed following three main malting stages (steeping, germination and kilning). Briefly, 60 g of dry grains were placed in glass recipients of 100 mL and washed under flowing water stream before steeping. The process was repeated per triplicate for every desired mycotoxin level and barley batch. Steeping stage consisted of alternated controlled cycles of water immersion and aeration in order to achieve moisture content of 45-48% by the end of the stage. The following order of cycles was applied: 13 hours of water immersion, 9 hours of aeration (all water from the previous stage was drained and barley grains were exposed to a gentle air flow in the thermoregulated chamber with a periodic mixing to facilitate water evaporation on grain's surface) and then another 4 hours of water immersion. The process was carried out at 10 °C in a thermoregulated chamber Memmert GmbH+ (Schwabach, Germany). At the end of steeping, the excess water was drained, and the samples were placed at 15 °C for germination during 96 hours. During the first 24 hours of germination, the grains were gently mixed and water was sprayed (40 cm^3/kg) every 4-5 hours, in order to avoid grain's surface dehydration. The grains were gently mixed daily to provide a good aeration and to ensure a good germination yield. Once the germination was over, all the samples were located in the drying chamber (Malting Plant "Malteria la Moravia SL", Bell-lloc d'Urgell, Spain), where four temperature levels were applied: 3 hours at 50 °C, 16 hours at 60 °C, 2 hours at 68 °C and 3 hours at 85 °C.

Sampling was performed after the following stages of malting process: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, then every 24 h of germination process (g24h, g48h, g72h and g96h) and finally, at the end of drying process (dry24h). Totally, twenty-seven recipients were prepared for each desired contamination level (9 sampling stages per replicate).

2.7 Sample preparation

All samples were dried (40 °C) and ground with IKA® A11 Basic (Darmstadt, Germany). For the extraction of DON, DON-3-G (DONPREP®) and ZEA (EASY EXTRACT® ZEARALENONE) specific immunoaffinity columns from R-Biopharm (Rhone LTD, Glasgow, Scotland) were used.

2.7.1 DON and DON-3-G extraction

Considering the finding of Zachariasova et al. (2012) that DONPREP® immunoaffinity columns are cross-reactive to DON-3-G, its extraction was performed simultaneously. Five grams of ground sample was mixed with 1 g of sodium chloride and 40 mL of Mili Q water

into a 250 mL Erlenmeyer flask, followed by 30 min stirring. Then, samples were centrifuged for 10 min at 1846 g. Supernatant was filtered through a glass microfiber paper filter (Whatman, Maidstone, UK) and 2 mL of the filtrate was passed through the column. The immunoaffinity column was then washed with 10 mL of bi-distilled water and the toxins were eluted with 3 mL of methanol HPLC grade (1.5 mL performing back-flushing and 1.5 mL for the final elution). Samples were then evaporated under a gentle nitrogen stream at 40 °C. Dry extract was resuspended in 0.5 mL of mobile phase (acetonitrile:methanol:water, 5:5:90, *v/v/v*), filtered with nylon filter (0.4 µm) and 50 µL were injected into HPLC-DAD system.

2.7.2 ZEA extraction

Five grams of ground sample was mixed with 25 mL of extraction solvent acetonitrile:water (75:25, *v/v*) and stirred for 30 min. Samples were centrifuged for 10 min at 1846 g and 10 mL of supernatant was mixed with 40 mL of Phosphate Buffer Saline at pH of 7.4 (PBS: 8 g of sodium chloride, 1.2 g of disodium hydrogen phosphate, 0.2 g of potassium di-hydrogen phosphate, 0.2 g of potassium chloride and 1 L of distilled water). The pH of the obtained extract was adjusted back to 7.4 with 2 M solution (80 g/L) of sodium hydroxide. The obtained 50 mL was passed through the immunoaffinity column which was afterwards washed with 20 mL of PBS. ZEA was eluted with 3 mL of acetonitrile (1.5 mL performing back-flushing and 1.5 mL for the definite elution). Samples were evaporated under a low nitrogen stream at 40 °C. The dry extracts were resuspended in 1 mL of acetonitrile:water (50:50, *v/v*) and 50 µL were injected into HPLC-FD system.

2.8 UHPLC analysis

2.8.1 DON and DON-3-G analysis

For DON and DON-3-G detection, an Agilent Technologies 1260 Infinity HPLC system (California, USA) coupled with an Agilent 1260 Infinity II Diode Array Detector (DAD) and a Gemini® C18 column from Phenomenex 150x4.6 mm (California, USA) with a particle size of 5 µm and a pore size of 110 Å was used for separation. Absorption wavelength was setup at 220 nm. The mobile phase was composed of methanol:acetonitrile:water (5:5:90, *v/v/v*) and set at a flow rate of 1 mL/min. The total run time was of 16 min.

2.8.2 ZEA analysis

For ZEA analysis, the same equipment as in 2.7.1 and same column were used but with an Agilent 1260 Infinity Fluorescence detector. The excitation and emission wavelengths were 274 nm and 455 nm, respectively. The mobile phase was acetonitrile:water (60:40, *v/v*) and a

pH adjusted to 3.2 with acetic acid. Flow rate was set at 0.6 mL/min. Total run time was 14 min.

2.9 Validation of analytical methods

Selectivity was checked by injecting 50 µL of standard solution for at least three times (150 µg/L), comparing retention time and peak resolution between injections. For linearity check, a calibration curve of at least seven concentration levels (10, 20, 30, 50, 100, 250, 500, 1000, 2000 µg/L for DON and 30, 70, 150, 300, 1000, 1500, 2000 µg/L of ZEA solutions) was prepared and injected into the system, generating a linear regression plotting solutions' concentration versus peak area. The standard solutions of DON-3-G injected to test for linearity represented ½ of DON concentrations in prepared solutions. Finally, precision was evaluated preparing blank barley samples spiked with DON (143.4, 750, 1075.5 and 1434 µg/kg), DON-3-G (50, 250 and 500 µg/L) and ZEA (35, 75, 150 µg/L) at several concentration levels and percentage recoveries were identified: 74 to 121 % for DON, 77 to 93 % for DON-3-G and 75 to 98 % for ZEA. For DON and DON-3-G % recovery determination, blank samples were spiked with both toxins simultaneously. The limit of detection (LOD) was considered as three times the signal to noise ratio (Table 2). Method performance was carried out according to Commission Regulation (EC) Nr. 401/2006 (European Commission , 2006).

2.10 Statistical analysis

ANOVA test was applied to assess the effect of the type of contamination and the initial mycotoxins concentration. *Ad hoc* multiple comparison Tukey HSD's test was also performed to locate more specifically the significant changes during malting process, using JMP Pro® 13 software (SAS Institute, New York, USA).

3 Results and discussions

3.1 Barley mycobiota

An important factor to be considered in the mycotoxins production in a cereal sample is the severity of *Fusarium* infection (Nielsen, Cook, Edwards, & Ray, 2014). Thus, with the purpose of the study of kernels mycobiota, the disinfection of kernels surface allowed to assess those microorganisms colonising the grain and not only being present on its surface, once excluded the ones on the surface. The main moulds found were identified as *Rhizopus* spp., *Penicillium* spp., *Aspergillus* spp. and on a lower extent *Alternaria* spp. in both samples with light distribution differences between the species. *Fusarium* was not found in any of the plated kernels. Considering that *Fusarium* is a pre-harvest contaminant and storage

conditions are harmful to it, this result is in accordance with the published literature (Beattie, Schwarz, Horsley, Barr, & Casper, Howard, 1998).

3.2 Fate of DON and DON-3-G during malting

There are several published studies on *Fusarium* mycotoxins' transfer from barley to malt (Kostelanska et al., 2009; Lancova et al., 2008), but they used artificially contaminated grains, either with standard mycotoxin solutions (Inoue et al., 2013) or with a mycotoxin producer *Fusarium* spore suspension (Habler et al., 2017; Lancova et al., 2008).

Firstly, the results on naturally contaminated barley will be presented. Table 3 shows the transfer of DON and DON-3-G throughout malting process for naturally contaminated barley. The obtained initial concentrations for DON were 407±54 and 676±304 µg/kg in levels 1 and 2, respectively; DON-3-G concentrations before the malting started were 64±40 and 170±123 µg/kg for the levels 1 and 2, respectively. As showed by previous studies, DON was partially washed out from barley grains during steeping due to its water solubility. However, its decrease achieved a lower extent compared to the results obtained by Lancova et al. (2008) in their work (90% of DON was washed out compared to the initial content): for naturally contaminated barley, in the present work, a significant decrease ($p<0.05$) of 22.4 % after 13h of water phase, reaching a 75.52 % decrease by the end of the process was recorded .

DON-3-G behaved significantly different depending on the initial concentration ($p<0.05$): a constant increase in DON-3-G concentration for low level of contamination of the naturally contaminated barley (Fig. 1a) was observed, but a variable trend in the high contamination level occurred in the steeping process.

Germination temperature and humidity, allow *F. graminearum* development and mycotoxin production (Medina et al., 2006; Ramirez, Chulze, & Magan, 2006), which could explain the initial increase in DON concentration at this stage, with respect to it level at the end of steeping, in all the studied scenarios, a peak concentration being achieved on the second day of the process (17 and 27.7 % increase at the 48 h of germination in the levels 1 and 2, respectively). No fungal growth was observed in naturally contaminated barley batches, which suggests DON release from the unextractable parts of the matrix. Worth noting, DON level was quite similar after 48h of germination whatever the initial contamination level, within the same contaminated batch, nevertheless its level was significantly lower compared to the initial contamination (t=0). DON-3-G levels also increased, however its increase was intensified by the end of the germination process, probably due to the continuous

accumulation of glucose molecules and intensification of enzymatic DON glycosylation reaction (Maul et al., 2012). Nonetheless, Lemmens et al., (2005), in their work, identified a close relationship between DON-3-G formation and the resistance of the grains variety to DON. DON's level decrease on the germination step was accompanied by an important increase in DON-3-G concentration at the same malting stage (from 20 to 147 % increase after the second day of germination, in the case of low level of contamination in the naturally infected barley and from 11.5 to 92.8 % increase in the batch with a higher contamination). The last 24 h of germination were characterized by a significant decrease in both DON and DON-3-G concentrations in all the samples compared to previous days of the process.

Kilning, as proven by previously reported studies, did not show any destructive effect on either DON nor DON-3-G levels in the case of naturally contaminated barley (Lancova et al., 2008). Moreover, an increase of mycotoxin concentration compared to the end of germination process was noticed in this batch, particularly for the low levels of contamination: 21.3 and 4.5 % increase in DON for low and high contamination levels, respectively; 107.3 and 33.2 % increase in DON-3-G concentration for low and high contamination levels, respectively.

The obtained initial concentrations for DON were 117±43 (level1), 165±52.4 (level2) and 168±88 µg/kg (level 3). DON-3-G was not detected (<LOD). The same decreasing trend of DON concentration occurred in the laboratory inoculated barley batch after steeping (approximately 34 % decrease), but only for the low contaminated batch and almost no changes were reported in the two other contamination levels (Table 4). This may be partially explained by DON washing from the grains with steeping water (Maul et al., 2012). The initial low DON-3-G concentration (<LOD) in laboratory inoculated batch and the moderate increment during steeping (which may result from the activation of grains enzymatic equipment), proves that its formation is a result of the start of enzymatic activity and is not of a fungal contamination.

Fungal growth during germination process in laboratory infected barley led to a greater DON concentration rise, especially in the samples with a lower level of contamination (9066, 3496.3 and 2671.6 % rise in the contamination levels 1, 2 and 3, respectively) (Table 4). Also, DON-3-G concentration raised following the same pattern as DON (2959.1, 1892.8 and 396.2 % for low, medium and high contamination levels). In laboratory inoculated barley, DON and DON-3-G concentrations variations behaved significantly different compared to the naturally contaminated batch. Only low levels of contamination showed a very similar behaviour to naturally contaminated barley (increase of DON and DON-3-G concentrations

by 63.5 and 2.1 %, respectively). After kilning, almost a 78 % decrease of DON-3-G was observed. Also, in the medium contamination level, there was a 24.7 % increase in DON content and a 13.4 % decrease in DON-3-G.

3.3 ZEA fate during malting

There is scarce information concerning the fate of ZEA during malting (Habschied, Šarkanj, Klapec, & Krstanović, 2011). A very similar trend was observed between the two levels of contamination for naturally contaminated barley (Table 5). ZEA initial concentrations obtained before malting were 38±34 (level 1) and 55±31 µg/kg (level 2) for the naturally contaminated batch, and 34±6, 110±47 and 194±97 µg/kg in laboratory contaminated batch for the levels 1, 2 and 3, respectively. More than 40 % decrease in ZEA concentration was obtained after removing the first steeping water (t=13h) in naturally contaminated barley. During the following steps of the steeping process, the decrease was not so important (48 and 72 % for low and high contamination levels, respectively). No significant differences, however, were observed.

Germination process was characterized by an increase of ZEA's concentration at the beginning of the process, with a peak at 48 h, followed by a decrease compared to ZEA's concentration before the beginning of germination. No visual fungal growth was noticed in naturally contaminated barley and the increase in ZEA's concentration to an almost same level as before malting process could be explained by a possible elution of ZEA from grain's matrix, which might take place, however there are no studies investigating ZEA's transfer through malting which would confirm this statement.

Except for low contaminated samples (decrease up to 64% in naturally contaminated barley), kilning process led to an increase in ZEA's levels present in barley compared to the initial concentration, with 64 % in the high level of contamination of naturally contaminated barley, although the changes were not significant. Considering the physical parameters of the kilning process (moderate change in temperature and water evaporation, see 2.5), infected grains could be still subjected to ZEA production by *F. graminearum* (Kostelanska et al., 2011).

ZEA concentration's evolution in the laboratory inoculated barley showed almost no change up after 48 h of germination (Table 5), where ZEA's concentration increased by 350, 134.3 and 40 % compared to the initial concentration in contamination levels 1, 2 and 3, respectively, although such increases were not significant.

4 Conclusion

The transfer of DON and ZEA through malting process was investigated in naturally contaminated barley. From the three malting stages (steeping, germination and kilning), the effect on the three mycotoxins had a similar tendency. It was confirmed, as in previous studies, that DON is washed out with steeping water (up to 75% of reduction). ZEA concentration remains stable during malting. DON contamination, on the contrary, is significantly reduced during malting process. An increase of DON-3-G levels was reported at the end of the process (277 % increase in the low level of contamination), however a decrease was registered for the level 2 of contamination (25% decrease). Considering the variability of the results, the abovementioned changes in DON-3-G concentration were not significant.

In the case of laboratory contaminated barley, on the contrary, malting led to a rise in DON concentration but without overpassing the legal limits for food for direct human consumption (750 µg/kg), except for the medium contamination level. DON-3-G level also increased (from <LOD up to 1179 µg/kg for the level 2 of contamination). ZEA was subjected to an almost two-fold increase in medium and high contamination levels, compared to its initial concentration, however the performed statistical analysis did not prove this increase significant.

In this study, a decrease in DON concentration was registered by the end of the process, nonetheless accurate barley analysis at the entry point and implementing good storage and production practices is highly important to ensure a safe final product. Also, the present work clarifies the fate of DON, DON-3-G and ZEA during the malting process and gives a better understanding of the properties of these compounds.

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6 References

Andrews, S., Pardoel, D., Harun, A., & Treloar, T. (1997). Chlorine inactivation of fungal spores on cereal grains. *International Journal of Food Microbiology*, *35*, 153–162.

Badr, A., Mu, K., Scha, R., Rabey, H. El, Effgen, S., Ibrahim, H. H., Pozzi, C., Rohde, W., Salamini, F. (2018). On the Origin and Domestication History of Barley (Hordeum

vulgare). *Genetic Engineering and Biotechnology Research Institute*, *17*, 499–510.

Beattie, S., Schwarz, P. B., Horsley, R., Barr, J., & Casper, Howard, H. (1998). The effect of grain storage conditions on the viability of Fusarium and deoxynivalenol production in infested malting barley. *Journal of Food Protection*, *61*, 103–106.

Beccari, G., Caproni, L., Tini, F., Uhlig, S., & Covarelli, L. (2016). Presence of Fusarium species and other toxigenic fungi in malting barley and multi-mycotoxin analysis by liquid chromatography-high-resolution mass spectrometry. *Journal of Agricultural and Food Chemistry*, *64*, 4390–4399.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. De, Haesaert, G., Karlovsky, P., Oswald, P. I., Seefelder, W., Speijers, G., Stroka, J. (2013). Masked mycotoxins: A review. *Molecular Nutrition and Food Research*, *57*, 165–186.

Freire, L., & Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189–205.

Habler, K., Geissinger, C., Hofer, K., Schüler, J., Moghari, S., Hess, M., Gastl, M., Rychlik, M. (2017). Fate of fusarium toxins during brewing. *Journal of Agricultural and Food Chemistry*, *65*, 190–198.

Habschied, K., Šarkanj, B., Klapec, T., & Krstanović, V. (2011). Distribution of zearalenone in malted barley fractions dependent on Fusarium graminearum growing conditions. *Food Chemistry*, *129*, 329–332.

Hietaniemi, V., Rämö, S., Yli-Mattila, T., Jestoi, M., Peltonen, S., Kartio, M., Sieviläinen, E., Koivisto, T., Parikka, P. (2016). Updated survey of Fusarium species and toxins in Finnish cereal grains. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *33*, 831–848.

Inoue, T., Nagatomi, Y., Uyama, A., & Mochizuki, N. (2013). Fate of Mycotoxins during Beer Brewing and Fermentation. *Bioscience, Biotechnology, and Biochemistry*, *77*, 1410–1415.

Kostelanska, M., Hajslova, J., Zachariasova, M., Malachova, A., Kalachova, K., Poustka, J., … Krska, R. (2009). Occurrence of deoxynivalenol and its major conjugate, deoxynivalenol-3- glucoside, in beer and some brewing intermediates. *Journal of*

Agricultural and Food Chemistry, *57*, 3187–3194.

Kostelanska, M., Zachariasova, M., Lacina, O., Fenclova, M., Kollos, A. L., & Hajslova, J. (2011). The study of deoxynivalenol and its masked metabolites fate during the brewing process realised by UPLC-TOFMS method. *Food Chemistry*, *126*, 1870–1876.

Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of Fusarium mycotoxins and 'masked' deoxynivalenol (deoxynivalenol-3-glucoside) from field barley through malt to beer. *Food Additives & Contaminants: Part A*, *25*, 732–744.

Lemmens, M., Scholz, U., Berthiller, F., Dall 'asta, C., Koutnik, A., Schuhmacher, R., Adam, G., Buerstmayr, H., Mesterházy, Á., Krska, V., Ruckenbauer, P. (2005). The Ability to Detoxify the Mycotoxin Deoxynivalenol Colocalizes With a Major Quantitative Trait Locus for Fusarium Head Blight Resistance in Wheat. *Molecular Plant-Microbe Interactions*, *18*, 1318–1324.

Maul, R., Müller, C., Rieß, S., Koch, M., Methner, F. J., & Irene, N. (2012). Germination induces the glucosylation of the Fusarium mycotoxin deoxynivalenol in various grains. *Food Chemistry*, *131*, 274–279.

Medina, Á., Valle-Algarra, F. M., Mateo, R., Gimeno-Adelantado, J. V., Mateo, F., & Jiménez, M. (2006). Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of Alternaria, Aspergillus and Fusarium. *International Journal of Food Microbiology*, *108*(2), 196–203.

Meussdoerffer, F. G. (2009). A Comprehensive History of Beer Brewing. In *Handbook of Brewing: Processes, Technology, Markets* (pp. 1–42).

Nielsen, L. K., Cook, D. J., Edwards, S. G., & Ray, R. V. (2014). The prevalence and impact of Fusarium head blight pathogens and mycotoxins on malting barley quality in UK. *International Journal of Food Microbiology*, *179*, 38–49.

Pascari, X., Ramos, A. J., Marín, S., & Sanchís, V. (2018). Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. *Food Research International*, *103*, 121–129.

Pestka, J. J. (2007). Deoxynivalenol: Toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology*, *137*(3–4), 283–298.

411 Piacentini, K. C., Savi, G. D., Pereira, M. E. V, & Scussel, V. M. (2015). Fungi and the
412 natural occurrence of deoxynivalenol and fumonisins in malting barley (Hordeum
413 vulgare L.). *Food Chemistry*, *187*, 204–209.

414 Pietri, a, Bertuzzi, T., Agosti, B., & Donadini, G. (2010). Transfer of aflatoxin B1 and
415 fumonisin B1 from naturally contaminated raw materials to beer during an industrial
416 brewing process. *Food Additives & Contaminants. Part A, 27*, 1431–1439.

417 Pitt, J. I., & Hocking , A. D. (1997). *Fungi and food spoilage. Second edition* . Blackie
418 Academic & Professional.

419 Ramirez, M. L., Chulze, S., & Magan, N. (2006). Temperature and water activity effects on
420 growth and temporal deoxynivalenol production by two Argentinean strains of Fusarium
421 graminearum on irradiated wheat grain. *International Journal of Food Microbiology*,
422 *106*, 291–296.

423 Schwarz, P. B., Casper, H. H., & Beattie, S. (1995). Fate and development of naturally
424 occurring Fusarium mycotoxins during malting and brewing. *Brewing Chemistry*, 121-
425 127.

426 Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova,
427 J. (2013). “Emerging” mycotoxins in cereals processing chains: Changes of enniatins
428 during beer and bread making. *Food Chemistry*, *136*, 750–757.

429 Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012).
430 Deoxynivalenol oligoglycosides: New “masked” fusarium toxins occurring in malt,
431 beer, and breadstuff. *Journal of Agricultural and Food Chemistry*, *60*, 9280–9291.

432 Zinedine, A., Soriano, J. M., Moltó, J. C., & Mañes, J. (2007). Review on the toxicity,
433 occurrence, metabolism, detoxification, regulations and intake of zearalenone: An
434 oestrogenic mycotoxin. *Food and Chemical Toxicology*, *45*, 1–18.

435

Table

Table 1: Rate of mycotoxins contaminated barley grains added to uncontaminated barley in the mixes prepared for micro malting

Barley batches	**Mycotoxins contaminated grains contained in the mix, %**		
	Level 1	*Level 2*	*Level 3*
Naturally contaminated	62	100	NP*
Laboratory infected	5.3	11.36	22.8

* NP=not performed;

Table

Table 2: Method performance parameters for deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-G) and zearalenone (ZEA)

Mycotoxin	LOD[1], µg/kg	Spiking levels, µg/kg	Replicates	Recovery±SD[2], %	RSD_r[3], %
DON	20	143.4	5	121.46±12.27	10.11
		750	7	105.6±6.32	5.99
		1075.5	7	74.12±3.36	4.54
		1434.0	5	82.93±0.91	1.10
DON-3-G	30	50	3	93.0±6.82	7.33
		250	5	80.76±13.54	16.76
		500	3	77.20±12.36	16.01
ZEA	2.5	35	5	96.07±11.02	11.47
		75	7	98.17±4.88	4.98
		150	5	75.18±4.43	5.90

[1]LOD – limit of detection; [2]SD – standard deviation; [3]RSD_r – relative standard deviation

Table

Table 3: Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) mean concentration (µg/kg) ±SD[1] at each malting stage in naturally contaminated barley.

Malting stage	Level 1, µg/kg		Level 2, µg/kg	
	DON	**DON-3-G**	**DON**	**DON-3-G**
T=0	407±54.1 a	64±40 a	676±304 a	170±123 a
S13h	316±118 ab	72±49 a	236±20 b	125±42 a
S22h	216±71 bc	108±76 a	172±25 b	70±26 a
S26h	209±80 bc	100±70 a	166±54 b	70±49 a
G24h	166±27 bc	117±43 a	195±61 b	89±50 a
G48h	245±18 abc	194±103 a	212±58 b	171±50 a
G72h	169±37 bc	183±81 a	140±10 b	228±89 a
G96h	130±36 c	173±62.5 a	113±19.4 b	71±94 a
Dry24h	217±70 bc	242±107 a	143±26.5 b	127±116 a

[1] SD=standard deviation; Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different); Samples: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, every 24 h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).

Table

Table 4: Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) average concentration (µg/kg) ±SD[1] at each malting stage in laboratory contaminated barley

Malting stage	Level 1, µg/kg		Level 2, µg/kg		Level 3, µg/kg	
	DON	**DON-3-G**	**DON**	**DON-3-G**	**DON**	**DON-3-G**
T=0	117±43 b	<LOD	165±52.4 b	<LOD	168±88 b	<LOD
S13h	70±36.5 b	34.8±32 b	101±7 b	<LOD	87±13 b	<LOD
S22h	50±10.6 b	<LOD	110±50 b	<LOD	146±117 b	67±44 c
S26h	40±8.9 b	38±38 b	127±37 b	53±34 a	166±11 b	103±38 c
G24h	151±24 b	37±36 b	155±24 b	35±33 a	168±11 b	125±18.5 bc
G48h	4079±2826 a	527±352 ab	5320±887 a	501±591 a	4363±1168 a	1073±826.6 ab

G72h	3639±1732 a	1175±580 a	4564±1578 a	1057±1155 a	4604±985 a	513±235 bc
G96h	144±157 b	255±368 b	677±554 b	1362±1259 a	1106±469 b	1621±395 a
Dry24h	235±55 b	261±85 b	844±372 b	1179±550 a	351±270 b	357±340 bc

[1] SD=standard deviation; [2] Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different). Samples: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, every 24 h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h); LOD= limit of detection: 20 and 30 µg/L for DON and DON-3-G, respectively.

Table

Table 5: Zearalenone's (ZEA) average concentration (µg/kg) ±SD[1] at each malting stage for both naturally contaminated and laboratory inoculated barley batches

Malting stage	Naturally contaminated barley		Laboratory infected barley		
	Level 1, µg/kg	*Level 2, µg/kg*	*Level 1, µg/kg*	*Level 2, µg/kg*	*Level 3, µg/kg*
T=0	38±34 a[2]	55±31 a	34±6 a	110±47 a	194±97 a
S13h	22±6.6 a	17.6±1.7 a	35±21.28 a	132±92 a	273±190 a
S22h	17.4±3 a	14.6±0.97 a	33±9 a	61±14.5 a	200±211 a
S26h	19.7±3.6 a	15.5±3 a	36±25.5 a	58±21 a	239±195 a
G24h	20±4.4 a	31±26 a	43±29 a	231±258 a	248±149 a
G48h	49±45 a	30±11.5 a	153±189 a	257±109 a	272±89 a
G72h	36±18 a	21±3.4 a	152±45 a	269±138 a	314±65 a
G96h	32±6 a	17.4±2.2 a	220±257 a	126±37 a	230±29 a
Dry24h	17.7±2.3 a	42±31 a	28±7.4 a	156±90 a	431±166 a

[1] SD=standard deviation; [2] Connecting letters for Tukey HSD test (levels not connected by the same letter within a column are significantly different). Samples: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, every 24 h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).

Figure

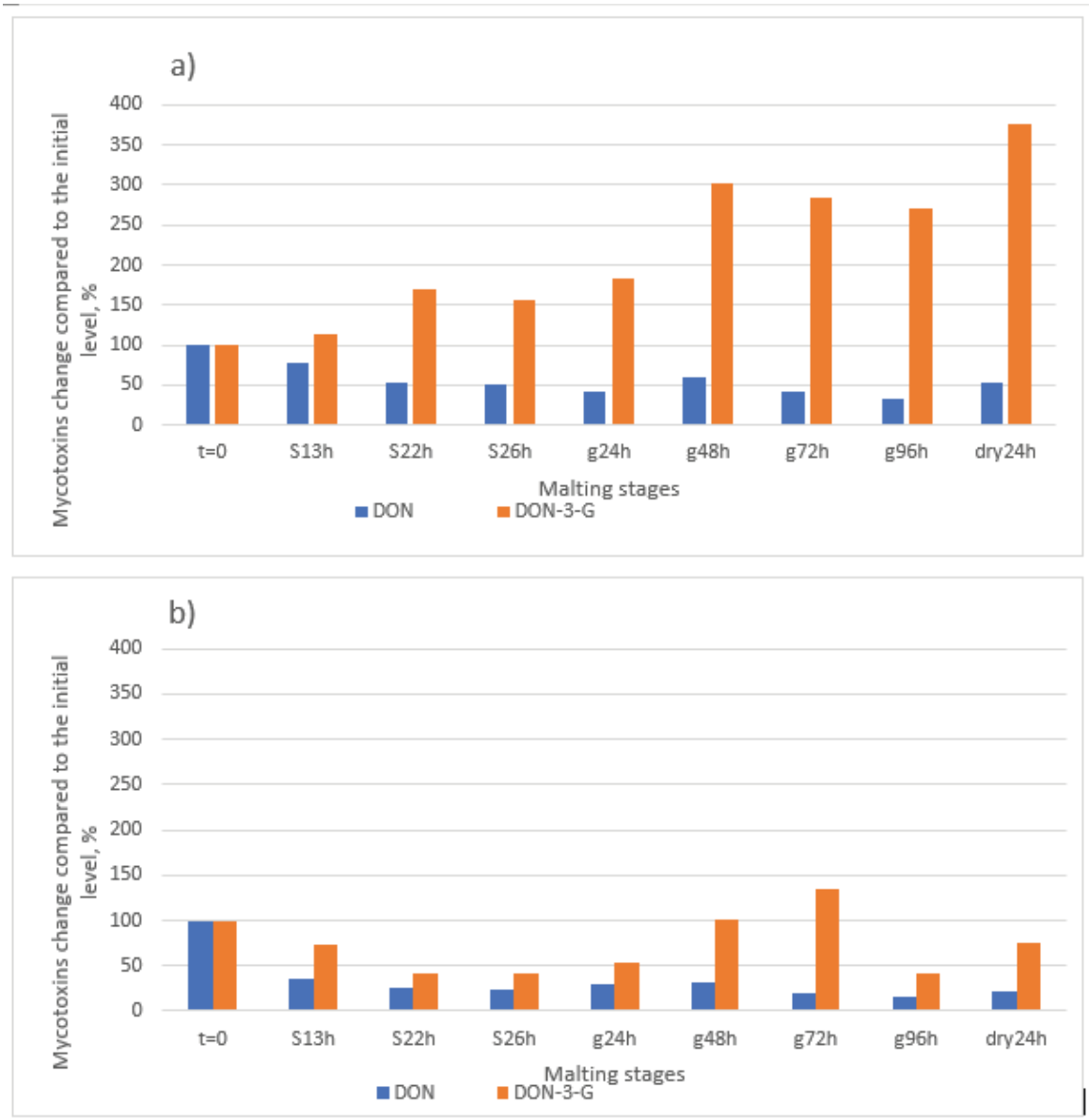

Figure 1: Evolution of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) concentrations in naturally contaminated barley at a) level 1 and b) level 2 of contamination during malting process. Samples: right after barley mixing (t=0); 13 h (s13h), 9 h (s22h) and 4 h (s26h) of steeping, every 24 h of germination process (g24h, g48h, g72h and g96h) and at the end of drying process (dry24h).